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# INFLAMMATORY-LIKE REACTION IN THE TUNIC OF *CIONA INTESTINALIS* (TUNICATA). II. CAPSULE COMPONENTS

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## Abstract

An inflammatory-like process is initiated in the tunic of *Ciona intestinalis* by particulate agents and soluble proteins. It includes the induction of a capsule in the form of a whitish disc in the tunic and which isolates injected materials. Histologically, the capsule structure shows a large number of cells collected around the injection wound. Vesicular cells release an unidentified amorphous substance which mixes with mucopolysaccharide and protein contents of typical "granule-packed cells" to form the capsule matrix. The vacuolated epithelial cells, lining the inner zone of the tunic, can release an unidentified substance which participates in matrix production. Lymphocyte-like cells can be present and transitional cells may differentiate into the vesicular cells. Large refringent granule cells, and compartment cells which can contain refringent material, were found inside the wound and are thought to release the glycoprotein substances. The degranulation of eosinophil granulocytes occurs in the same period. The capsule induced by soluble proteins lacks "granule-packed cells" while fine granular material, mucopolysaccharide in nature, is layered on the epithelium of the capsule. Observations of early stages of capsule formation show granular and hyaline amoebocytes, probably phagocytes, which quickly surround the foreign material (*e.g.*, erythrocytes) followed by transitional cells and, finally, vesicular cells, which can form an incipient capsule within 24 hours. Encapsulation in *Ciona intestinalis* is a cellular response which includes the mechanisms for tunic production in order to isolate the inflamed tissue while the foreign material is disrupted and the injection wound repaired.

## Introduction

In tunicates, encapsulation is a chronic inflammatory-like response which appears, in the body wall, to isolate natural invaders (Bresciana and Lützen, 1960; Monniot, 1963; Dudley, 1968), experimentally inserted objects (Smith, 1970; Anderson, 1971), or bacteria (Thomas, 1931). Vacuolated blood cells, predominantly morula cells, are responsible for these reactions (Wright, 1981; Wright and Ermak, 1982).

The ascidian, *Ciona intestinalis,* reacts by cellular responses toward foreign materials inserted into the tunic (Wright and Cooper, 1975; Parrinello *et al.*, 1976, 1977). In previous papers we have shown that a capsule can envelop the particulate or soluble injected materials by cell infiltration and glycoprotein secretions (Parrinello *et al.*, 1976, 1977). In some specimens the response can induce local tissue damage which seems to be independent of encapsulation. The tunic injury depends on the dose and nature of the irritant, involves eosinophil granulocytes, and shows lysosomal content release by various degranulation mechanisms (Parrinello and De Leo, in prep.).

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Abbreviations: SE = sheep erythrocytes; PBS = phosphate-buffered saline (0.01 *M* pH 7.4 phosphate buffer containing 0.15 *M* NaCl); BSA = bovine serum albumin; Hc = *Octopus vulgaris* hemocyanin; Hb = bovine hemoglobin; PAS = periodic acid Schiff.

The *C. intestinalis* capsule is a whitish disc around the injected material and is visible through the transparent tunic. Each treated specimen can react in various degrees to the foreign matter and the intensity of the response is dependent upon both the nature and the dose of the eliciting agent (Parrinello *et al.*, 1984). Preliminary histological observations of capsules obtained after erythrocyte injection showed numerous univacuolated cells and packed granular materials in the inflamed area. In the present paper the histopathology of the capsules produced after injection of particulate or soluble agents is described, and data are also reported on the stages in differentiation and role of some capsular components.

## Materials and Methods

Adult *Ciona intestinalis* L. specimens (~10–12 cm in length) were collected from Palermo harbor. Animals with tunics free of marine matter were selected and maintained at 15–18°C in glass tanks containing adequately aerated sea water.

### *Injection of animals*

The following particulate material suspensions were prepared in phosphate-buffered saline at pH 7.4 (PBS): $5 \times 10^7$/ml sheep erythrocytes (SE), 2 and 20 mg/ml colloidal carbon (G. Wagner, Hannover, Lot C 11/1431 A), and 10% (v/v) sheep red cell membranes prepared according Davis and Bakerman (1972). The response to a more complex cellular system was investigated by injecting *C. intestinalis* oocytes collected from several specimens, washed, and suspended in PBS ($1.0–1.2 \times 10^3$ oocytes/ml). Bovine serum albumin (BSA) (Sigma), bovine hemoglobin (Hb) (Sigma), and *Octopus vulgaris* hemocyanin (Hc) (kindly supplied by Dr. G. Nardi, Zoological Station, Naples) were used at the concentration (2 mg/ml) which is known (Parrinello *et al.*, 1984) to produce a visible capsule in the *C. intestinalis* tunic. A 0.2 ml volume was injected, using a syringe and 27-gauge needle, into the tunic under the cuticle in the region of the gut loop. Control specimens were injected with 0.2 ml PBS.

### *Light microscopy*

Portions of the tunic from the injected area, whether showing a capsule or not, and the corresponding region of the untreated or PBS-injected specimens, were fixed in 70% ethanol and embedded in paraffin wax or paraplast. Histochemical reactions were performed on 7 μm sections using techniques reported by Beccari and Mazzi (1966), Luna (1968), and Ganter and Jollès (1970). Mallory's trichrome and hematoxylin-eosin stains were employed in general cytological studies to identify tunic zones and cells, Millon's reaction was used to detect proteins, the periodic acid-Schiff (PAS) test for polysaccharides, and 1% alcian blue stain (pH 1.0 and 2.5) for mucopolysaccharides. Pickworth's method with benzidine for hemoglobin was used.

Some of the cell types in the inflamed tunic correspond to cells described by other authors using light and electron microscopy techniques on normal tissues of *C. intestinalis.* Their definitions are: (1) lymphocyte, blood cell (3–5 μm) with a high nuclear:cytoplasmic ratio (Millar, 1953; Rowley, 1981); (2) vesicular cell, blood and tunic cell (5–7 μm) with a single large vacuole occupying most of the cell and forcing the discoid nucleus to one side, the shape can be signet-ring-like (Millar, 1953); (3) morula cell, round or elliptical shaped cell (6–8 μm) found in both the blood and the tunic, containing several globules around the cell periphery, the nucleus often cannot be distinguished (De Leo *et al.*, 1981; Rowley, 1981); (4) large refringent granule cell, tunic cell (4–6 μm) with a single large refringent granule which occupies almost all the volume of the cell, the nucleus is displaced in a cytoplasmic polar cup

(*cfr.* De Leo *et al.*, 1981); (5) granular amoebocyte, blood cell (13.4 × 7.3 μm, mean values) showing cytoplasm with a variable number of granules and occasional vacuoles; protoplasmic extensions are evident (Rowley, 1981); (6) hyaline amoebocyte, blood cell (6.7 × 15.9 μm, mean values) showing cytoplasm with a number of large vacuoles, is highly amoeboid, and often has lobose pseudopodia (Millar, 1953; Rowley, 1981), both types of amoebocytes were also found in the tunic (Millar, 1953; De Leo *et al.*, 1981); (7) mantle epithelium cells, they are polygonal cells which form a flattened single layered epidermis immediately under the tunic, their cytoplasm is weakly stained and their nuclei has scattered chromatin blocks, a vacuole can occupy part of the cell (Millar, 1953).

## Results

### *The tunic of untreated and PBS-injected specimens*

To compare the normal with treated tunics, serial transverse sections from pharingeal and gut-loop regions of five untreated specimens were first examined.

The tunic consists of fibers (polysaccharides and proteins) embedded in an amorphous ground substance (acid mucopolysaccharides and low concentrated proteins) and cells (De Leo *et al.*, 1977; Patricolo and De Leo, 1979; De Leo *et al.*, 1981). The tissue is organized in a gelatinous "inner zone" and a dense "outer zone" showing an external glycoprotein cuticle and an internal single-layered epithelium.

The cell types (see Methods) and their frequencies (numbers based on counts of each optical field at 315×) can vary.

Large refringent granule cells and morula cells, usually observed in the outer layer, can occasionally be found in the inner zone; they were rare in three specimens.

Granulocytes, round, elliptical, or amoeboid (granular amoebocyte) in shape, can be scattered variously throughout the tunic: 2–3 cells per optical field were found in three specimens, while 10–15 cells were near the epithelium in two.

Vesicular cell, signet-ring-like, show a single large vacuole full of unstained material which reduces the cytoplasm to a peripheric strip. They were rare in two specimens and absent in one; 5–9 cells per optical field were found in two specimens.

Lymphocytes were only found (2–4 cells per optical field) in a few tunic areas near the epithelium of two specimens.

The cells of the epithelium show a vacuole containing unstained material.

To investigate the mechanical effect of the liquid injection, histological sections of tunic fragments were examined at different time intervals after 0.2 ml PBS injection. A tunic reaction was never observed. Initially the saline injection produced a wound in the tunic matrix which then healed; in fact it was not observed in two specimens fixed after 4–6 hours. No unusual distribution or frequency of cell types were found in this area even when two different tunic fragments were examined 12 hours and 5 days after the injection, respectively.

### *Capsule structure*

Following injection of particulate material or proteins into the tunic of *C. intestinalis* a capsule (1.5–3.0 cm wide) can appear within 2–8 days (Fig. 1a). The appearance time is variable depending on dose, nature of the eliciting agent, and, probably, on the animal lot used in each experiment (Parrinello *et al.*, 1984).

*Capsule induced by particulate materials.* Figure 1 (b, c) shows transverse sections of the tunic seven days after injection of *C. intestinalis* oocytes. The injected material is contained in a tunic wound, while cells infiltrate the inflamed area apparently

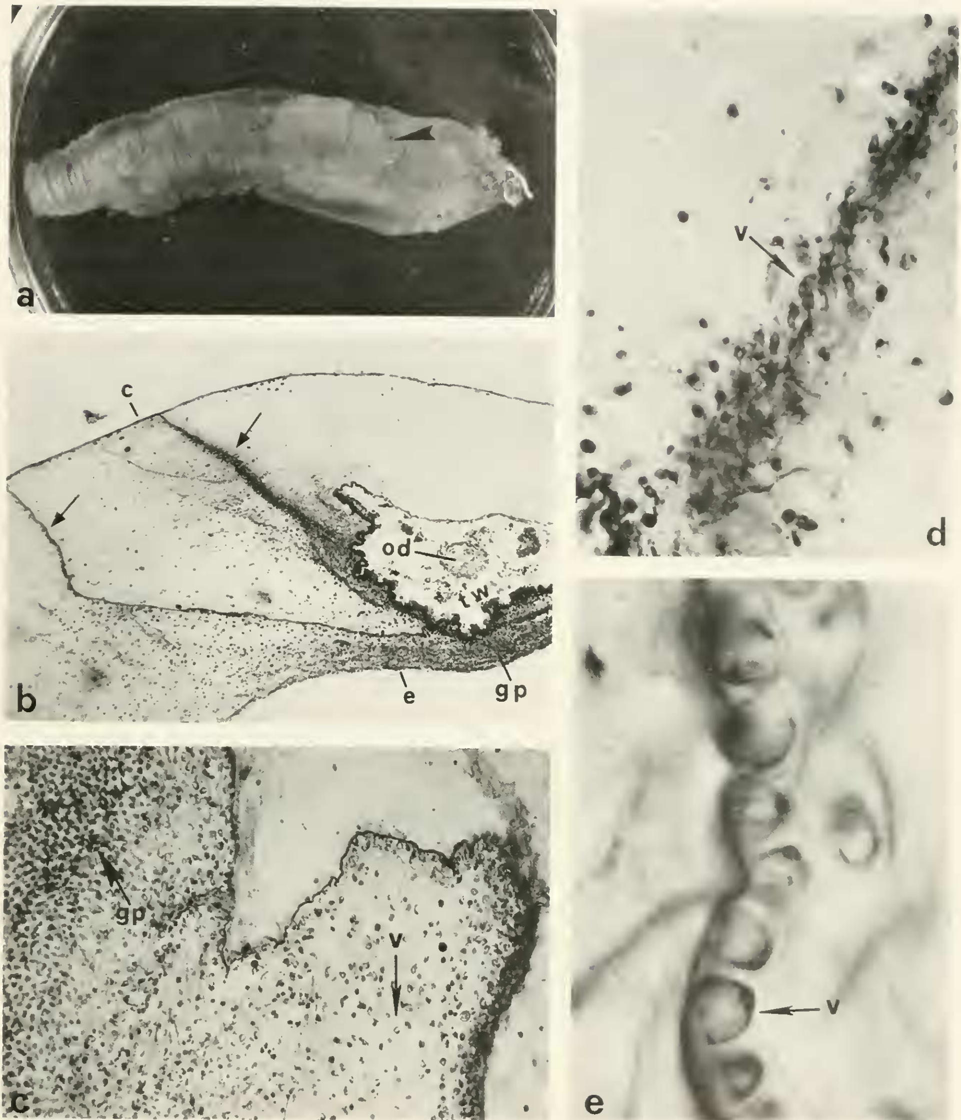


FIGURE 1. Encapsulation of ascidian oocytes injected into *Ciona intestinalis* tunic. (a) Capsule seven days after injection (arrowhead). (b–e) Transverse sections of the tunic containing capsule (Mallory's stain); (b) arrows indicate the lined up cells which envelop the foreign material, 40×; (c) closer view of the capsule 470×; (d) amoeboid vesicular cells (v) forming the capsular outline, 630×; (e) vesicular cells which release vacuolar content to form an encapsulating strip, 1420×. c, cuticle; e, epithelium; gp, granule-packed cells; od, oocyte debris; tw, tunic wound.

originating from the tissue under the epithelium to enclose the injured tunic up to the cuticle (Fig. 2).

Histological observations of tunic fragments from five specimens injected with $1 \times 10^7$ SE and two specimens injected with oocytes, showed that cells accumulated around the wound in large bands in the tunic, from the epithelium to the foreign

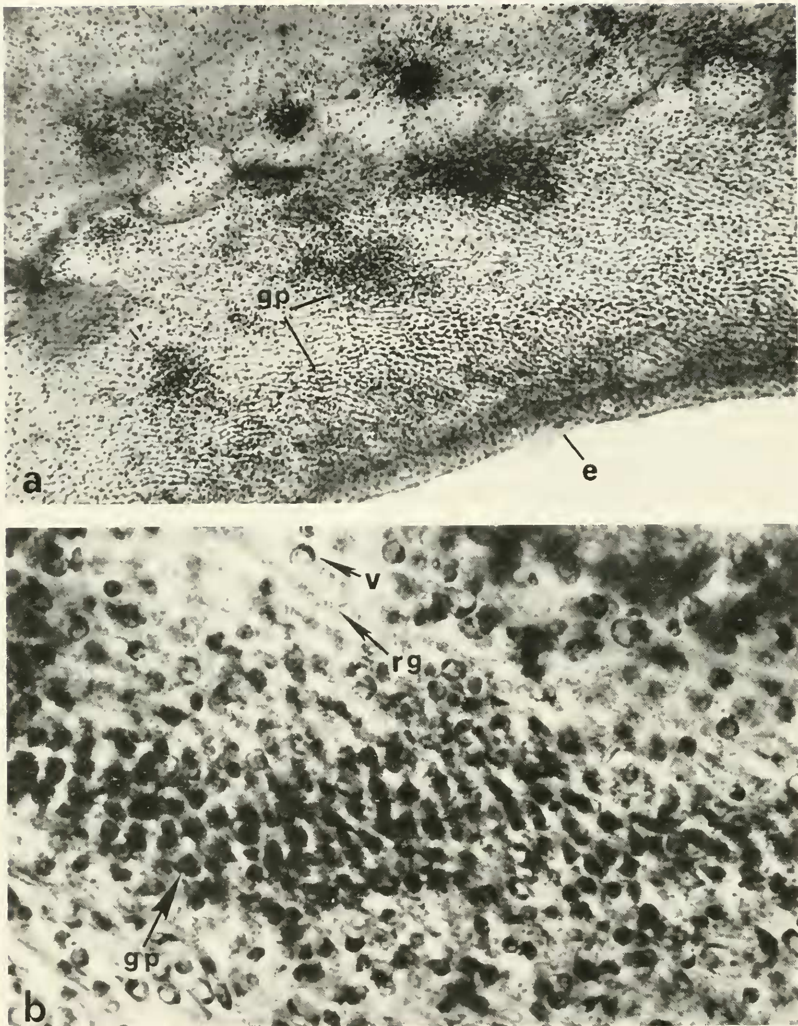


FIGURE 2. Capsule transverse sections seven days after sheep erythrocyte injection into *Ciona intestinalis* tunic (Mallory's stain). (a) Tunic inner zone lined by epithelium (e), 95×. (b) Closer view showing granule-packed cells (gp), released granular material (rg) and vesicular cells (v), 570×.

material. They are called "granule-packed cells" because their cytoplasm is completely filled with strongly aniline blue (Mallory's trichrome) stained fine granules which mask the nucleus (if present); their diameters range from 2.1 to 5.6 $\mu$m (Fig. 3a–c). The granules are positive to Millon, PAS, and alcian blue reactions. In some cells they are less packed and more easily distinguished, in other cells the granular material apparently dissolves while cells are elongated and stretch out releasing their contents into the tunic matrix.

In three specimens injected with erythrocytes, granule-packed cells were found in close contact with the epithelium and up to the inner border of the wound. In longitudinal sections they were arranged in 2–3 concentric layers around the wound.

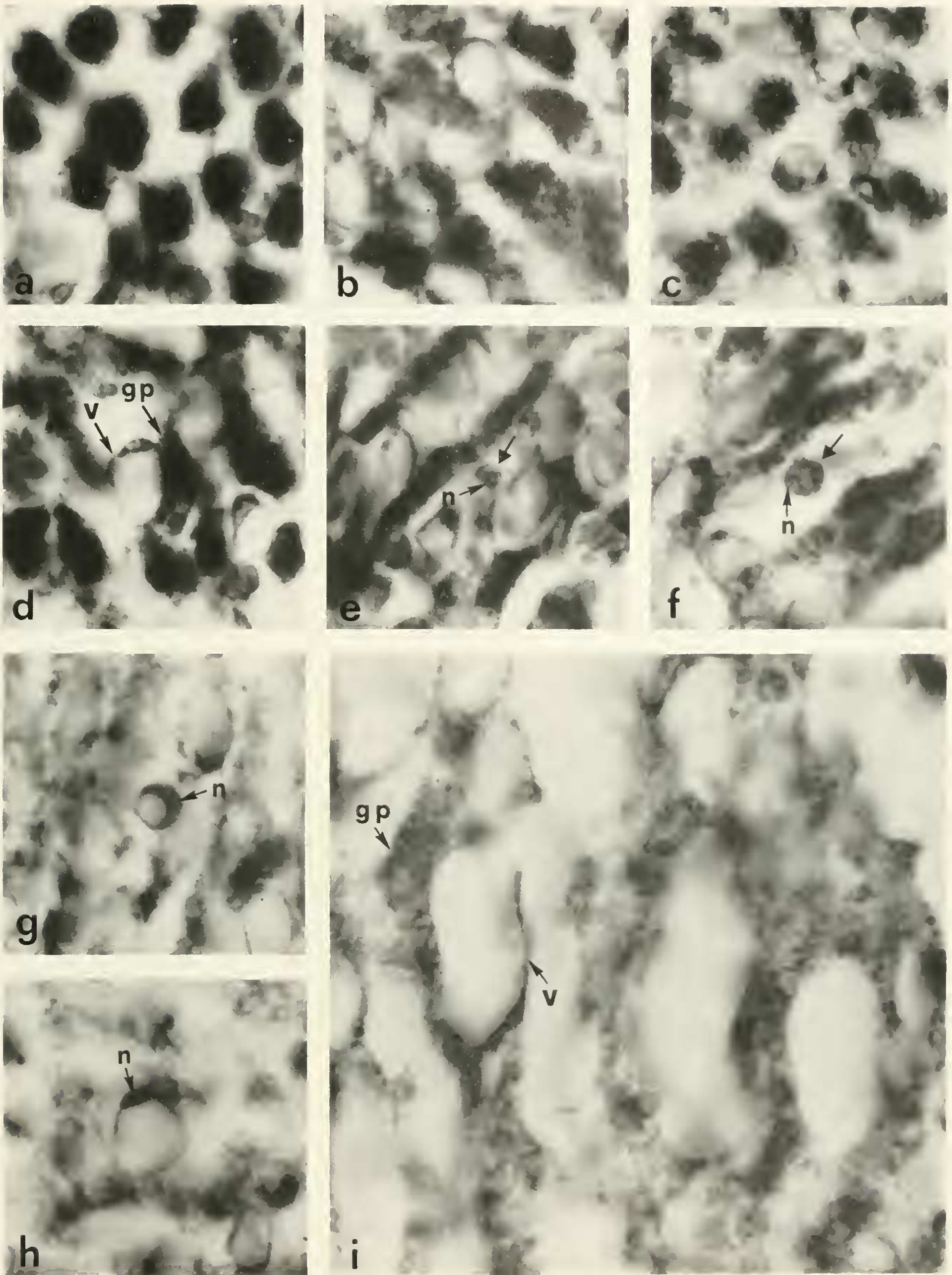


FIGURE 3. Capsular components seven days after erythrocyte injection into *Ciona intestinalis* tunic, 1420× (Mallory's stain). (a–c) Various features of granule-packed cells. (d) Vesicular cells (v) and granule-packed cells (gp) in close contact. (e) Lymphocyte (arrow) among vesicular cells and elongated granule-packed cells. (f) Transitional cell (arrow). (g) Immature vesicular cell (arrow). (h) Large vesicular cells releasing amorphous material. (i) End stages of granule-packed cells and vesicular cells. n, nucleus.

Vesicular cells filled the reacting tunic particularly at the inner edge of the wound. They range in size from 3.5 to 7.1 μm and vary in appearance according to their stage of development. These cells can assume an amoeboid shape and fall into line to form the capsular outline and encapsulating strip, some release their amorphous contents by dissolving a portion of their membrane (Fig. 1d, e). The vacuolar substance was negative with Mallory's stain, PAS, Millon, and alcian blue reactions.

Granule-packed cells and vesicular cells can be in close contact; their membranes apparently dissolve and their contents mix (Fig. 3d, i). Both cell types appear to contribute to the production of capsular substance. In some zones, the granular material has a streaked appearance due to ghosts of the large vesicular cells.

Small lymphocytes (2.1–3.4 μm) with an aniline blue slightly cytoplasm (Fig. 3e), are present in the reacting tunic.

Spherical cells, 3.2–4.5 μm in diameter, with an eccentric or central nucleus and aniline blue staining cytoplasm are considered transitional cells. They are frequent among the granule-packed cells and are numerous near the epithelium as far as the inner edge of the wound. Figure 3 (e–g) shows the presumptive stages in the vesicular cell differentiation.

The cells of the mantle epithelium (9.2–11.3 μm, in surface view), show vacuoles 3.5–7.4 μm wide containing an amorphous substance which is negative to Mallory's trichrome, PAS, Millon, and alcian blue histochemical reactions. Large vacuoles can release their contents into the tunic matrix by dissolving a portion of their membranes. Multi-layered epithelium was observed in some capsule transverse sections (Fig. 4).

Granulocytes (6.2–7.6 μm) are distributed along the edges and inside the wound; they contain eosinophil granules 0.6–0.8 μm in diameter and can degranulate, releasing the latter by cell membrane dissolution. In wounds produced by erythrocyte injection, the intensely stained material distributed along the edges makes it difficult to identify the granulocytes.

Large round cells (7.5–8.5 μm), probably phagocytes, containing vacuoles full of aniline blue-staining material, are frequently found at the edges of the wound. These vacuoles occupy the cytoplasm and contain substances which are PAS and Millon positive but alcian blue negative. The nucleus is indistinguishable (Fig. 5a).

Large refringent granule cells (4.5–6.4 μm) are numerous in the wound. A single large orange-stained granule occupies almost all of the cell (Fig. 5b). Cells with a smaller refringent granule show more evident semilunar cytoplasm. Other cells release their granule by dissolving a portion of the cell membrane. These last two cell types are not frequent in the tunic wound containing oocytes.

Compartment cells (6.3–7.9 μm) are characterized by 2–4 vacuoles separated from each other by cytoplasmic partitions (Fig. 5c); they are similar to those described in the blood of other ascidians (*cfr.* Wright, 1981). The vacuolar contents can be slightly stained by aniline blue or appear as yellow stained refringent inclusions; in some cells a prominent refringent inclusion can occupy a large part of the cytoplasm (Fig. 5d). The nucleus is not visible and the cell outlines are often obscure (Fig. 5e, f). Free yellow refringent granules (2.1–2.8 μm) can be found (Fig. 5g). Large numbers of compartment cells and free granules populate the wound.

The specimens injected with stromata (1% or 10%) or colloidal carbon (2 or 20 mg/ml) never showed an evident capsule. However histological investigations revealed some capsular components in the injected area. Within 6–7 days vesicular cells in various differentiation stages were distributed in the tunic matrix between the epithelium and the inner edge of the wound. Round phagocytes full of deeply aniline blue-stained material or carbon masses, and large refringent granule cells are scattered

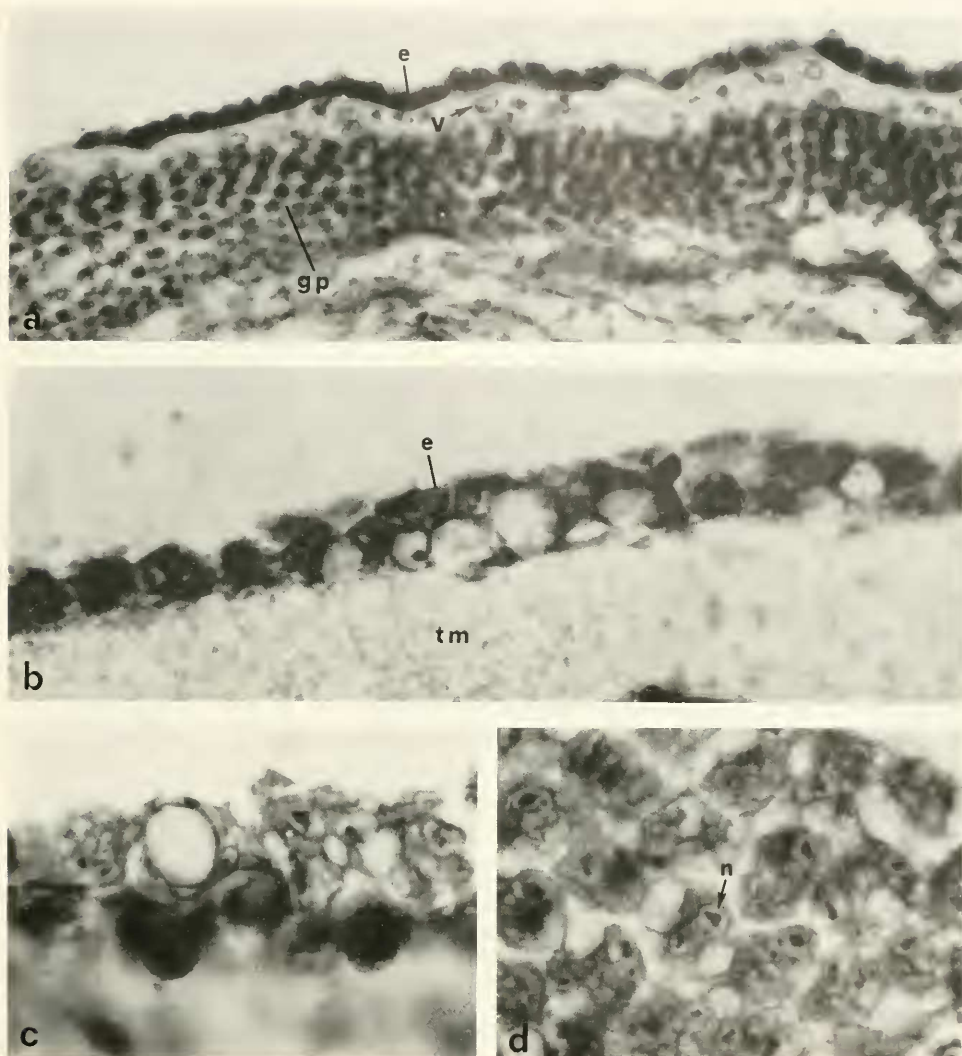


FIGURE 4. Epithelium and inner zone of *Ciona intestinalis* tunic after sheep erythrocyte injection (Mallory's stain). (a) Transverse sections showing single layered epithelium (e) and granule-packed cells (gp), 570×. (b, c) Active epithelium releasing vacuolar content, 1420×. (d) Surface view of the epithelial cells, 1420×. v, vesicular cell; n, nucleus; tm, tunic matrix.

among the foreign materials. When the highest doses were used these cells were more numerous.

*Capsule induced by soluble proteins.* Histological transverse sections of capsules obtained 6 days after 0.4 mg protein (BSA, Hc, Hb) injection, showed that the tunic around the wound is populated with vesicular cells varying in differentiation stages. Inside the wound, aniline blue-stained large round cells and large refringent granule cells are frequent; granulocytes are settled on the inner edge, where they can degranulate.

Fine granular materials (PAS, Millon, and alcian blue positive) were layered on

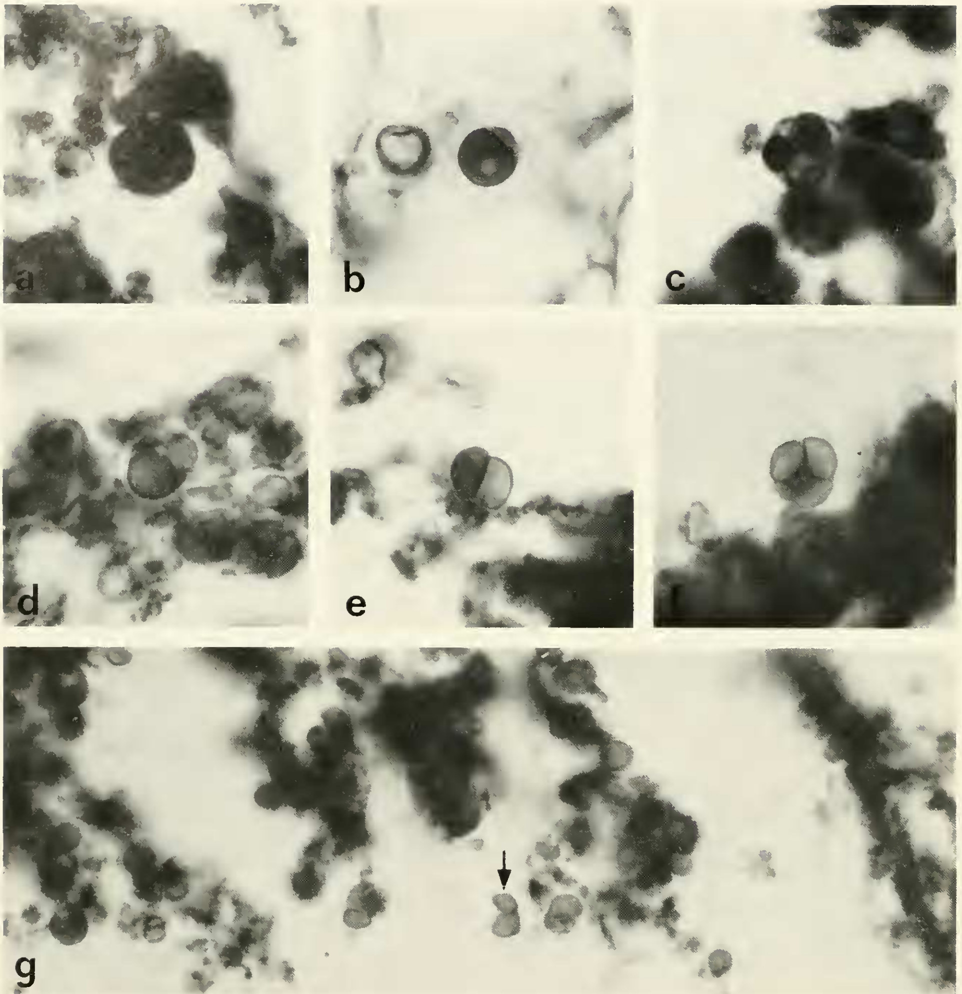


FIGURE 5. Cells inside the tunic wound seven days after erythrocyte injection; erythrocyte debris is indistinguishable, 1420× (Mallory's stain). (a) Large round phagocyte. (b) Large refringent granule cell. (c) Compartment cell. (d–f) Compartment cell with refringent inclusions. (g) Free refringent globules (arrow) inside the wound.

the epithelium forming a band which gradually decreased in thickness toward the capsule margin.

*Early stages in the tunic reaction*

Histological study of the tunic showed that rapid cellular responses follow erythrocyte injection. After 2–4 hours vesicular cells in various differentiation stages predominate near the epithelium. Aniline blue-stained large round phagocytes were scattered among the erythrocytes; the latter formed large masses at the inner edges of the wound.

In tunics fixed after 12–16 hours (Fig. 6a, c–e) many hyaline and granular amoebocytes (7.1–11.2 × 2.3–3.5 $\mu$m) formed a band along the inner wound edge and

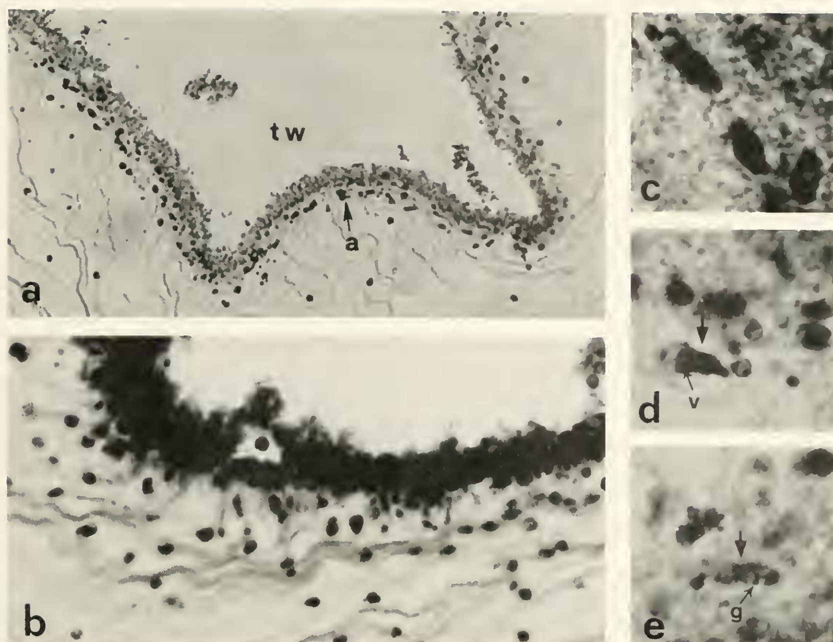


FIGURE 6. Early response in the tunic reaction of *Ciona intestinalis* after erythrocyte injection. (a, b) Transverse sections showing tunic wound (tw) and amoebocyte (a) infiltration. (a) Twelve hours after injection, 160×. (b) Twenty-four hours after injection, 390×. (c) Benzidine-positive amoebocytes, 980×. (d) Hyaline amoebocyte (arrow), 980×. (e) Granular amoebocyte (arrow), 980×. g, granule; v, vacuole. a, b, d, e: Mallory stained.

showed lobose pseudopodia. Some vesicular cells lined up forming an encapsulating strip while others filled the tunic matrix and may be amoeboid in shape. Large refringent granule cells are numerous inside the wound and are also present in the inner tunic layer below the wound. Nodules of transitional cells are observed near the epithelium.

In tunics fixed after 24 hours, the above cells are more numerous. On the wound inner edge, the cells are full of aniline blue-stained material (Fig. 6b) while the erythrocyte masses are not easily distinguished.

After 48 hours many compartment cells, with vacuoles containing yellow refringent material alone were found inside the wound. Granule-packed cells were also present in the tunic while large vacuoles characterized the epithelial cells.

At 12 and 24 hours the large round cells inside the wound and the amoebocytes were positive to the benzidine histochemical reaction for hemoglobin (Fig. 6c). Tests carried out at 48 hours and 6 days were negative.

The tunics from specimens which 6–7 days after SE injection did not show an apparent capsule, presented in the injection site the same components found in the early stages (12–48 hours) of the capsule development. Low general cell frequency and few granule-packed cells characterize these tunic areas.

The capsule increases in thickness and 12–20 days after $5 \times 10^7$ SE injection, forms a protuberance of the tunic and a decreased number of cells. The wound was reduced, probably by coalescence of the edges which appear partially free of cells and material. Encapsulating strips, when present, contained few vesicular cells.

A quick response followed stromata or colloidal carbon injection. Vesicular cells

formed thin encapsulating strips in the tunic fixed after 24–48 hours. Small carbon masses were in the round phagocytes inside the wound. Hyaline and granular amoebocytes with carbon inclusions, formed a band along the inner wound edge. Granule-packed cells or free granular material were never observed in the tunic.

## Discussion

*Ciona intestinalis* is capable of recognizing non-self materials and reacts by cellular responses. Foreign agents inserted into the tunic, including tunic tissue allografts, are removed from the body (Reddy *et al.,* 1975; Wright and Cooper, 1975; Parrinello *et al.,* 1977). These reactions are considered to form part of the internal defense system. They show characteristics of inflammatory-like processes including phagocytosis, encapsulation, and tissue damage.

Encapsulation is a response elicited by both corpuscular material and soluble proteins (Parrinello *et al.,* 1984). The nature and dose of the foreign agent determine the strength of the response and influence the structure of the capsule which is visible in the most reactive specimens. Early stages of encapsulation also occur in the tunic of apparently non-reactive animals; the absence of granule-packed cells or granular material lining the active epithelium could account for the non-appearance of the capsule.

The removal of the foreign material and tunic debris may be rapidly effected by phagocytes. These cells correspond reasonably well to the blood hyaline and granular amoebocytes described by Rowley (1981). They may begin the tunic reaction by performing non-self recognition. The nature of the cell contents in the responses to various agents suggests that the large round cells inside the wound could also be phagocytes. The removal of large masses may be facilitated by a lysosomal mechanism dependent on the eosinophil granulocyte degranulation. Large numbers of these cells were found in the wound containing oocytes, where the resultant degraded material may induce further inflammation (*cfr.* Hirschhorn, 1974; Gleisner, 1979). Such activity may also lead to tunic injury (Parrinello *et al.,* 1984).

Lymphocytes can infiltrate the inflamed tunic, however their quantity is not important to capsule composition. The transitional cells are numerous. They are a first stage in which stem cells differentiate to produce capsule cells. From morphological evidence the presumptive stages in vesicular cell differentiation are as described in the blood by Millar (1953). Lymphocytes and transitional cells as well as granular and hyaline amoebocytes may originate from the blood or from the nodules of hemopoietic tissue situated in the pharingeal wall and around the gut loop (Ermak, 1976).

An unidentified amorphous substance contributes to the manufacture of the capsule matrix. It is contained in large vacuoles of mantle epithelium cells and vesicular cells. It is released by vacuole and cell membrane dissolution. Vesicular cells rapidly fill the inflamed area and line up in a continous layer, isolating the injured tissue. In this respect encapsulation may involve the mechanisms that construct or regenerate the tunic. Enlargement and vacuolization of epithelial cells and cell types ("cell with a small acidophil vacuole" and "phagocyte univacuolated"), probably corresponding to differentiation stages of vesicular cells, have been described by Pérès (1948) in the tunic regeneration of *C. intestinalis.* Moreover, in the ascidian tunic, epithelial cells take up the glucose used in ground substance production. In *C. intestinalis* this monosaccharide is used in the manufacture of acid mucopolysaccharides, and may be incorporated into the tunic fibers (Robinson *et al.,* 1983). Evidence for the cellulose nature of this capsular material is not available, but histochemical evidence suggests

that it collaborates with mucopolysaccharides in tunic matrix formation. This was particularly evident when the fate of the vesicular cells and the granule-packed cells was observed. The packed fine granules consisting of mucopolysaccharides gradually dissolve and apparently mix with the vesicular cell products. The origin of the granule packed cells and fine granular material is unknown. They might have an epithelial origin because they frequently appear in close contact with the epithelium.

Large refringent granule cells have been described in the regeneration of the tunic as occasionally occurring across the epithelium (Pérès, 1948) and usually present just below the cuticle. They may be involved in the production of this glycoprotein layer (De Leo *et al.,* 1981) and may produce the glycoproteins inside the wound and contribute to the healing by cuticular material formation.

Compartment cells could be stages in morula cell development as proposed for the corresponding blood cells in other ascidian species (Endean, 1960; Kalk, 1963; Smith, 1970). The absence of mature morula cells in the inflamed area could depend on the activity of lysosomal enzymes which interrupt cell development and induce granule release. It is known that morula cells congregate and break down at the edge of wounds produced by extirpating fragments of *Halocynthia aurantium* tunic (Smith, 1970). Moreover, they form the capsule which surrounds glass fragments inserted into the tunic of *Molgula manhattensis* (Anderson, 1971).

Differences in the cell composition of the capsules apparently depend on the nature and size of the irritant. Apart from the granule-packed cells and round phagocytes, the other cells are also found in the normal or PBS-injected tunic. They are variably distributed and definitely less frequent. As yet the origin of the capsule cells is unknown.

The contribution of naturally occurring humoral factors in the ascidian non-self recognition mechanisms is unclear. However, hemagglutinins are probably not involved in the erythrocyte tunic encapsulation of *C. intestinalis* (Parrinello *et al.,* 1984).

In summary, the tunic reaction of *C. intestinalis* is a response consisting of several processes: (1) non-self recognition by phagocytes which can intervene in early stages of the reaction; (2) degranulation of eosinophil granulocytes which can disrupt large foreign material, probably by lysosomal mechanisms which in some cases may cause tissue damage (Parrinello *et al.,* 1984); (3) tunic matrix substance production with formation of a thick capsule to isolate the inflamed area; and (4) cuticular glycoprotein production to heal the tunic wound.

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